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α-Adrenergic regulation of secretion of mouse saliva rich in nerve growth factor

(bioassay/radial immunodiffusion)

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ABSTRACT **Nerve growth factor has been quantified by both bioassay and radial immunodiffusion in mouse saliva elicited by several secretagogues. The concentrations by bioassay of nerve growth factor in both epinephrine- and norepinephrine-induced saliva (3400 and 900 μg/ml, respectively) are higher than reported in any other source. In contrast, the concentrations of nerve growth factor in isoproterenol- and pilocarpine-induced saliva are relatively low (17 and 2 μg/ml, respectively). The specific activity of the salivary nerve growth factor was 41, 36, 2, and 0.6 μg/mg of protein in secretions elicited by epinephrine, norepinephrine, pilocarpine, and isoproterenol, respectively. Salivation after administration of either epinephrine or norepinephrine was completely inhibited by the α-adrenergic blocker, phenoxybenzamine. These results suggest that the release of saliva rich in nerve growth factor is primarily regulated through α-adrenergic receptors.**

The submaxillary gland of male mice is the richest known source of nerve growth factor (NGF) (1–3). Venom of numerous species of snakes (4, 5) and the submaxillary glands of female mice (2, 3) are also rich sources of NGF. Since snake venom is the secretory product of the venom gland, the phylogenetic homologue of the mammalian salivary gland, early investigators also looked for NGF in mouse saliva. Levi-Montalcini and Cohen (6) reported that pilocarpine-induced mouse saliva contained detectable levels of biologically active NGF, but estimated the concentration to be at least 5000 times lower than in salivary gland extracts. Those studies only examined saliva induced by a parasympathetic secretagogue, despite the fact that salivary secretions are also elicited by activity of the sympathetic nervous system. Such adrenergic secretions differ from cholinergic saliva in both protein concentration and composition (7, 8).

Several lines of evidence suggest that NGF might be secreted from the submaxillary gland after adrenergic stimulation. This growth factor is concentrated in the convoluted granular tubules (9, 10) with a number of other proteins that are secreted by the gland: (*i*) renin (or isorenin) (11, 12), (*ii*) esteroproteases (13, 14), and (*iii*) epidermal growth factor (EGF) (15, 16). Of these proteins, both the esteroproteases and EGF are secreted in saliva elicited by adrenergic agonists (14, 16). In addition, Pasquini *et al.* (17) have demonstrated that NGF, EGF, and esteroprotease activity are all associated with the same intracellular granules isolated from the mouse submaxillary gland.

In the present paper and in an earlier abstract (18), we report exceedingly high NGF concentrations in salivary secretions elicited by epinephrine or norepinephrine, but not by isoproterenol or pilocarpine. The high NGF concentrations in both epinephrine- and norepinephrine-induced salivas have been quantified by both bioassay and radial immunodiffusion. NGF release was inhibited by the α-adrenergic blocker, phenoxybenzamine. Thus, a secretion rich in NGF that is both biologically active and immunologically reactive is selectively released by the action of adrenergic agonists on α-receptors within mouse salivary glands.

MATERIALS AND METHODS

Saliva was collected from 10- to 16-week-old male mice. The animals were anesthetized with pentobarbital, 60 mg/kg. Salivation was usually induced by the intraperitoneal injection of secretagogue. The secretagogues and ranges of doses for each were: pilocarpine, 0.1–0.8 mg/kg; epinephrine, 2.0–6.6 mg/kg; norepinephrine, 0.7–2.5 mg/kg; and isoproterenol, 0.25 mg/kg. In a few cases about one-tenth the intraperitoneal dose of secretagogue was injected under the sheath of connective tissue covering the submaxillary gland. In all experiments, pooled salivary secretions were collected in a microcapillary tube placed between the tongue and the floor of the mouth over a 45-min period after injection of the secretagogue. (The ducts from the submaxillary and sublingual glands open into the buccal cavity under the tongue.) Immediately after collection, the saliva was frozen at −40° until the assays were performed.

The inhibitors phenoxybenzamine and propranalol were injected at 5 mg/kg into the jugular vein 30 and 60 min, respectively, prior to administration of the secretagogue.

The NGF bioassay was performed using cultures of para-

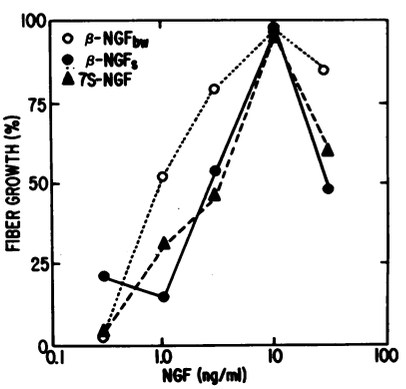

FIG. 1. Standard bioassay curves for different samples of β-NGF and 7S-NGF using 13-day chick sympathetic ganglia. Of the concentrations examined, 10 ng/ml yielded maximal fiber outgrowth. The NGF concentrations that would have produced optimal fiber outgrowth, as estimated by the curve-fitting method of Fenton (22), were 6 ng/BU, 7 ng/BU, and 7 ng/BU for β-NGF supplied by Dr. Shooter (β-NGF$_s$), β-NGF supplied by Burroughs-Wellcome (β-NGF$_{bw}$), and 7S-NGF, respectively. NGF concentrations refer to the final culture medium. Values on the ordinate give percent fiber growth in terms of the maximal halo width obtained in each experiment (0.23–0.25 mm in all cases). Details of the bioassay are given in *Materials and Methods*.

Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; BU, biological unit.

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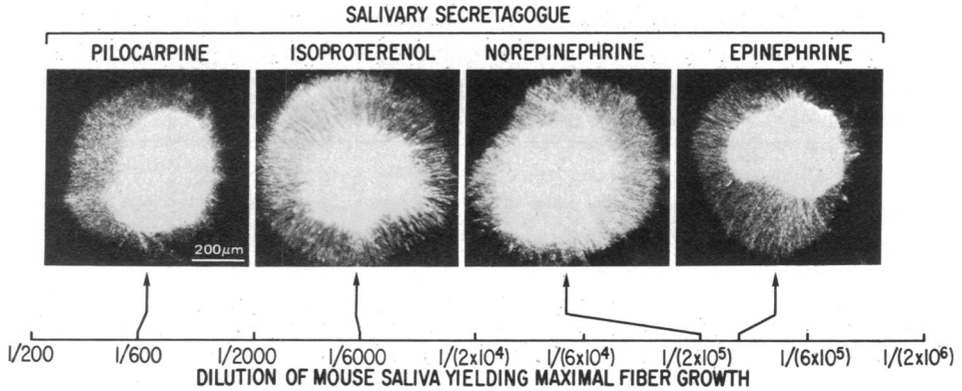

FIG. 2. Nerve fiber outgrowth by 13-day chick sympathetic ganglia at optimal concentrations of four samples of mouse saliva elicited by different secretagogues. Dilution values refer to total dilution of a given saliva in the culture medium. Thus, NGF in norepinephrine-induced saliva was sufficiently concentrated that the equivalent of 5 nl of undiluted saliva in 1 ml of culture medium caused maximal fiber outgrowth. These photographs were taken at low magnification with dark-field illumination. Details of the bioassay are given in *Materials and Methods*.

vertebral sympathetic ganglia from 13-day chick embryos after a modification (19) of the original method of Levi-Montalcini *et al.* (20). Microcapillary tubes (Drummond Scientific Co., Broomall, Pa.) were used for all dilutions and were discarded after a single use in order to avoid any carry-over of NGF (21). Five microliters of five or more dilutions were added to dishes containing four ganglia in 1 ml of culture medium. The bioassay was quantified by determining the mean width of the halo of nerve fibers surrounding each ganglion after 24 hr *in vitro* (19). The exact dilution that would have produced maximum halo width was calculated by the simplified curve-fitting method of Fenton (22). Replicate analyses of standard β-NGF solutions gave a specific activity of 7.0 ± 0.4 ng/biological unit ($n = 5$). Thus, one can conclude with a 95% certainty that a single measurement on an unknown will not differ from the population mean by more than ±29%.

Both β-NGF and 7S-NGF (23, 24) were used as standards in the bioassay. Samples of β-NGF and 7S-NGF were kindly supplied by Dr. Eric Shooter, Depts. of Genetics and Biochemistry, Stanford University. β-NGF was also obtained from Burroughs Wellcome, Co., Research Triangle Park, N.C. All preparations gave mean specific activities of approximately 7 ng of protein per biological unit (BU), as defined by Varon *et al.* (23). Typical bioassay results for each type of NGF are shown in Fig. 1. The specific activity of β-NGF as determined by our method (7 ng/BU) is close to that obtained by others (approximately 10 ng/BU; refs. 23, 25, and 26). Thus, our bioassay system using 13-day sympathetic ganglia has essentially the same sensitivity to β-NGF as that using 8-day sensory ganglia (23). In addition, our observation that β-NGF and 7S-NGF have similar specific activities confirms similar reports (23, 24).

β-NGF was also assayed by radial immunodiffusion (27). Five microliters of 5-fold concentrated antiserum to β-NGF† (Burroughs Wellcome Co.) were evenly spread on each gel disc 2 hr before addition of an aliquot (3 μl) of either β-NGF or a dilution of mouse saliva. The outer diameter of the immunoprecipitin ring corresponding to β-NGF was measured after 24 hr. Immunological identity between β-NGF and the salivary antigen was demonstrated by formation of a dumbbell-shaped immunoprecipitin band surrounding two closely spaced wells (4 mm, center to center), one containing saliva and the other β-NGF (1.5 μg in 3 μl), in an antiserum-impregnated gel slab (10–40 μl on a 1.0 × 1.5 cm gel).

Protein concentrations of salivary secretions were determined by the method of Lowry *et al.* (28), using bovine serum albumin (fraction V, 96–99% albumin; Sigma Chemical Co., St. Louis, Mo.) as a standard.

Statistical evaluations are given as means ± the standard error of the mean, and the number of samples is given in parentheses.

RESULTS

All samples of mouse saliva elicited by the autonomic agonists used in this study were capable of stimulating nerve fiber outgrowth from cultured sympathetic ganglia when added at an appropriate dilution (Fig. 2). The quality and quantity of nerve fiber growth at optimal concentrations were in all cases equivalent to that obtained using purified β-NGF (7 ng/ml). In the experiment shown in Fig. 2, the dilution at which optimal fiber outgrowth was obtained varied by up to 500-fold between samples of saliva elicited by different secretagogues.

The concentration of both bioassayable NGF and total protein in mouse saliva varied greatly depending on the autonomic agonist (Table 1). Epinephrine-induced salivary secretions had 77 times as much protein and 1375 times as much NGF as did pilocarpine-induced secretions. Thus, the specific activity of NGF in mouse saliva elicited by the adrenergic agonist, epinephrine, was 17 times that in saliva elicited by the cholinergic agonist, pilocarpine.

NGF and protein concentrations were also measured in mouse saliva elicited by isoproterenol and norepinephrine (Table 1) in order to determine whether the epinephrine-induced secretion of NGF was due to action on α-adrenergic or β-adrenergic receptors in the salivary glands. The β-adrenergic agent, isoproterenol, elicited saliva containing seven times as much NGF and 30 times as much total protein as found in pilocarpine-induced saliva. Saliva elicited by the α-adrenergic agent, norepinephrine, contained 320 times as much NGF and 22 times as much total protein as pilocarpine-induced saliva. Thus, the specific activity of NGF in saliva elicited by norepinephrine is 15 times that found in pilocarpine-induced saliva and equal to that found in epinephrine-induced salivary secretions (Table 1).

The ability of adrenergic agonists to elicit saliva was also studied in the presence of blocking agents selective for α- and β-receptors. Phenoxybenzamine, a potent blocker of α-ad-

† This antiserum also contains a significant pool of antibodies against the α-subunit of 7S-NGF even though it is said to have been prepared using purified β-NGF as the antigen. These data will be presented elsewhere.

Table 1. Concentration and specific activity of NGF in salivary secretions elicited by various autonomic agonists

Autonomic agonist	NGF concentration (μg/ml)*	Protein (μg/ml)	Specific activity (μg of NGF/ mg of protein)
Epinephrine (10) [α + β-adrenergic]	3300 ± 900	76,000 ± 13,000	41 ± 3
Norepinephrine (4) [α-adrenergic]	770 ± 300	22,000 ± 10,000	36 ± 11
Isoproterenol (5) [β-adrenergic]	17 ± 3	29,000 ± 2,100	0.6 ± 0.1
Pilocarpine (7) [cholinergic]	2.4 ± 0.8	990 ± 160	2.4 ± 0.5

The numbers of samples are shown in parentheses. The receptors primarily stimulated by each agonist are indicated by square brackets. All values are means ±SEM.

* NGF concentrations were determined by bioassay using 7 ng/BU as determined for purified β-NGF (see *Materials and Methods*).

renergic receptors, inhibited salivation after injection of either epinephrine or norepinephrine but had no effect on salivation elicited by isoproterenol (Table 2). Those samples of saliva elicited in the presence of phenoxybenzamine that were large enough to permit bioassay ($n = 3$) contained 23 ± 9 μg/ml of NGF and 35 ± 9 mg/ml of protein, giving a specific activity of 0.6 ± 0.1 μg of NGF per mg of protein. Thus, saliva elicited by either epinephrine or norepinephrine in the presence of phenoxybenzamine was similar to that elicited by the β-adrenergic agonist, isoproterenol (Table 1). Propranolol, a selective blocker of β-adrenergic receptors, completely blocked salivation after administration of isoproterenol ($n = 5$). Thus, epinephrine and norepinephrine appear to act through α-adrenergic receptors while isoproterenol appears to act through β-adrenergic receptors.

Norepinephrine- and epinephrine-induced salivary secretions were also examined by radial immunodiffusion. Two circular immunoprecipitin bands were found under the conditions used in this study. The outer ring was narrow and showed complete immunological identity with the α-subunit of 7S-NGF (24). The inner ring was broad and showed complete immunological identity with β-NGF, which also formed a broad immunoprecipitin ring. A standard curve was prepared for the radial immunoassay of salivary secretions with purified β-NGF (Fig. 3). Salivary concentrations of β-NGF were then estimated by measuring the outer diameter of the β-NGF-like immunoprecipitin band formed by different dilutions of saliva. Eight samples of adrenergically elicited saliva were assayed by both radial immunodiffusion and bioassay (Table 3). Both methods demonstrated that α-adrenergically elicited saliva contains a high concentration of NGF, but the values determined by radial immunodiffusion were only 40% of those estimated by bioassay.

The relative contribution made to the pooled salivary secretions by each of the salivary glands is not known. The size of these glands in adult male mice [submaxillary, 178 ± 10(7) mg per pair; sublingual, 23 ± 1(6) mg per pair; parotid 48 ± 3(7) mg per pair] suggests that a major contribution might come from the NGF-rich submaxillary glands. This is supported by determinations made on saliva elicited by injection of very small doses of various secretagogues under the sheaths covering the submaxillary glands. Epinephrine and pilocarpine elicited samples of saliva having specific activities of 37 and 1 μg of NGF per mg of protein, respectively. These values are not significantly different from those obtained after systemic administration of epinephrine and pilocarpine (36 and 2.4 μg of NGF per mg of protein, respectively, Table 1).

Table 2. Effect of phenoxybenzamine on salivation after administration of adrenergic agonists

Agonist	Volume of saliva (μl)	
	Control	Phenoxybenzamine
Epinephrine [4 mg/kg]	108 ± 14(14)	2 ± 2(5)
Norepinephrine [1.5 mg/kg]	119 ± 13(8)	0.7 ± 0.2(6)
Isoproterenol [0.25 mg/kg]	40 ± 7(4)	39 ± 10(6)

The numbers of samples are shown in parentheses. The agonist was administered intraperitoneally, at the dosage level given in brackets, 1 hr after intravenous injection of phenoxybenzamine at 5 mg/kg. All values are means ±SEM.

DISCUSSION

The results presented in this paper demonstrate that nerve growth factor is secreted in the saliva of male mice. The specific activity of biologically active NGF is surprisingly high in all types of mouse saliva (Table 4). The NGF specific activity of both epinephrine- and norepinephrine-induced saliva far exceeds that of either male mouse submaxillary gland homogenate or snake venom (Table 4). Even pilocarpine- and isoproterenol-induced salivas contain biologically active NGF of relatively high specific activity (Table 4), although the NGF concentration in such secretions is rather low (Table 1).

Data gathered by radial immunodiffusion demonstrate that immunologically reactive NGF is present in high concentrations in α-adrenergically elicited mouse saliva (Table 3). In comparison with determinations by radioimmunoassay, the concentrations in epinephrine- and norepinephrine-induced sali-

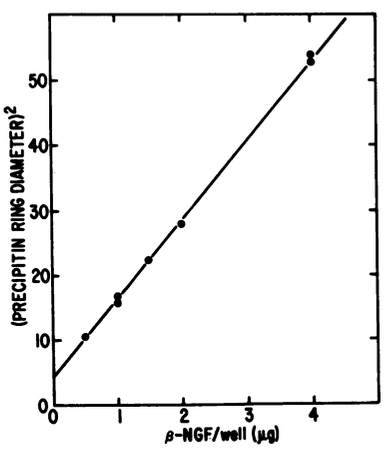

FIG. 3. A standard curve for radial immunodiffusion prepared with purified β-NGF. The square of the precipitin ring diameter in mm increased linearly with the amount of β-NGF in the well. The standard curve intersects the ordinate at 4 mm^2, which equals the square of the diameter of the antigen-containing well. Procedural details are given in *Materials and Methods*.

Table 3. A comparison of NGF concentrations in adrenergically elicited mouse saliva as determined by bioassay and by radial immunodiffusion

Secretagogue	β-NGF (μg/ml) Bioassay	β-NGF (μg/ml) Immunodiffusion	Bioassay/Immunodiffusion
Norepinephrine	1033 ± 312	385 ± 49	2.5 ± 0.5
Epinephrine	3534 ± 810	1455 ± 343	2.5 ± 0.5

Values represent means ±SEM (n = 4). The values shown for (bioassay/immunodiffusion) are the means ±SEM of the individually determined ratios. Procedural details are given in *Materials and Methods*. The salivary secretions assayed in this table were not included in Table 1.

vary secretions are equal to or greater than those in any other source (2, 31, 32). The quantitative difference observed between bioassay and radial immunodiffusion (Table 3) is not entirely unexpected as each measures a different parameter. Thus, bioassay provides an estimate of *total* NGF protein since all of the molecular species of NGF [β-monomers, β-dimers, (i.e., 2.5S and β-NGF), and 7S-NGF] have similar specific activities (Fig. 1 and refs. 24 and 26) and would be expected to have additive effects on growth. On the other hand, radial immunodiffusion only measures β-NGF-like immunologically reactive antigens and would not measure, for example, the α- and γ-subunits in 7S-NGF.

Calculation of the total amount of secreted NGF suggests that a major portion of the growth factor in the salivary glands can be released into salivary secretions. The saliva elicited by epinephrine, norepinephrine, and isoproterenol contained 51,000, 13,000, and 97 BU, respectively (Tables 1 and 2). It is estimated that the submaxillary glands contain at least 62,000 BU of NGF‡; this is likely to be too low as others have presented evidence for the presence of an inhibitor of biological activity in the crude submaxillary gland homogenate (23). The results nevertheless suggest that a considerable fraction of the NGF normally found in the submaxillary gland may be secreted in saliva after administration of epinephrine and norepinephrine.

Three observations suggest that the secretion of NGF-rich mouse saliva is regulated by action on α-adrenergic receptors. First, salivary secretions elicited by the adrenergic agonist epinephrine had a 17-fold higher specific activity of NGF than those secretions elicited by the cholinergic agonist pilocarpine (Table 1). Second, saliva induced by the α-adrenergic agonist norepinephrine had a 60-fold greater specific activity than saliva obtained after administration of the β-adrenergic agonist, isoproterenol (Table 1). Third, the α-adrenergic blocker phenoxybenzamine, inhibits secretion of NGF-rich saliva after administration of either epinephrine or norepinephrine (Table 2). Thus, agents having α-adrenergic agonist activity appear to release selectively an NGF-rich saliva. Other proteins that are secreted from mouse salivary glands by α-agonists include EGF (16) and a potent anticomplementary factor (33).

Salivary secretions elicited by either norepinephrine or isoproterenol have equal protein concentrations, but the specific activity of NGF is much higher in saliva elicited by the α-adrenergic agonist. These observations suggest that at least two distinct populations of protein-rich secretory granules must exist in the salivary glands. The release of the contents of one type of granule must be regulated through α-adrenergic receptors, while the contents of the other must be released by β-adrenergic agonists. Two populations of granules have recently been isolated from the mouse submaxillary gland (17). One population contained amylase while the other contained NGF, EGF, and esteroprotease. The secretion of the amylase-containing granules appears to be regulated through β-receptors (34), while release of EGF in saliva is induced by the α-adrenergic agonist phenylephrine (16). These observations strongly support our hypothesis concerning the mechanisms involved in the selective release of NGF by α-adrenergic agonists.

We have recently found that NGF is present in high concentration in saliva elicited by electrical stimulation of the preganglionic sympathetic nerve trunk (data not presented). This observation, along with those presented in this paper, suggest that the high concentrations of NGF in α-adrenergically elicited mouse saliva might have some physiological significance.

‡ This estimate uses the value of 1648 BU/mg of submaxillary gland protein (ref. 23; value is the average of those given on line 4 of Table 4), the protein content of the glands [0.21 ± 0.02 (six animals) mg of protein per mg of wet weight], and the mean weight of the gland pair (178 mg).

Table 4. A comparison of NGF specific activities of various materials as determined by bioassay

Material	Specific NGF activity (BU/mg of protein)	Ref.
β-NGF, highly purified	143,000	This paper
Saliva, adult male mouse, elicited by epinephrine	5,860	This paper
Saliva, adult male mouse, elicited by norepinephrine	5,140	This paper
Submaxillary gland homogenate, adult male mouse	1,430; 1,865*	23
Venom from *Vipera russeli* or *V. aspis*	810†	5
Submaxillary gland homogenate, adult male mouse	670	1
Venom from *Naja naja*, *Sepedon haemachates*, *Bitis gabonica*, or *V. ammodytes*	400†	5
Saliva, adult male mouse, elicited by pilocarpine	340	This paper
Venom from the Crotalidae family of snakes	205†	5
Submaxillary gland homogenate, adult female mouse	170	1
Saliva, adult male mouse, elicited by isoproterenol	86	This paper
Sympathetic ganglia homogenate, adult male mouse	~5	29
Serum, adult male mouse	0.2‡	30
Serum, adult female mouse	0.1‡	30

* Each value represents a separate experiment. They are only approximate, since the calculated number of BU per gland rose by as much as 30% during the initial stages of purification (23).

† These values have been calculated from data presented by Cohen in μg of venom per BU by assuming that 83% of the dried venom is protein. This estimate is based on information on yield of protein and bioactivity given for one type of venom (5).

‡ These values have been calculated by assuming a serum protein concentration of 70 mg/ml.

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